University of Alberta

NEUROPROTECTIVE EFFECTS OF GALANIN IN THE RAT CHOLINERGIC BASAL FOREBRAIN

By

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Abstract

Galanin (GAL) is a 29 amino acid peptide that is extensively distributed in the CNS and PNS. In Alzheimer's disease (AD) patients, GAL expression is elevated significantly and GAL was observed to hypertrophy and hyperinnervate the cholinergic neurons. However, the role of GAL in AD, detrimental or neuroprotective, remains controversial. We report GAL is consistently neuroprotective against β-amyloid (Aβ) toxicity in cholinergic basal forebrain (BF) neuronal cultures as judged by visual observation, MTT assay, live-dead cell assay and caspase 3 activation. We next demonstrated the neuroprotective effects of GAL were specifically directed towards cholinergic neurons in the BF cultures by double immunofluorescent staining for cleaved caspase 3 and VChAT. We further demonstrated that galanin receptor (GALR) 2 agonist ARM1896 could mimic the neuroprotective effects of GAL, indicating GALR2 may be involved in mediating this function. Finally we determined that GAL was also neuroprotective against glutamate (Glu)-induced excitotoxicity in the cholinergic BF neuronal cultures.

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List of Abbreviation

GAL	Galanin
AD	Alzheimer's disease
Aβ	Amyloid-β
GALR	Galanin receptor
Glu	Glutamate
BF	Basal forebrain
ChAT	Choline acetyltransferase
AChE	Acetylcholinesterase
ACh	Acetylcholine
MS	Medial septum
DBB	Diagonal band of Broca
NBM	Nucleus basalis of Meynert
APP	Amyloid precursor protein
m/nAChR	Muscarinic and nicotinic acetylcholine receptors
GAL-IR	Galanin-immunoreactivity
EPSP	Excitatory post-synaptic potential
NGF	Nerve growth factor
WT	Wild type
ABC	Avidin-biotin-peroxidase
NSE	Neuron-specific enolase
VChAT	Vesicular acetycholine transporter protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

TBS	Tris buffered saline
BSA	Albumin bovine serum

Chapter 1

Introduction

1.1 Neurochemical and neuropathological aspects of Alzheimer's disease (AD)

AD is the most common form of progressive senile dementia. The primary neuropathological features from the brains of AD patients include extracellular amyloid-β (Aβ) peptide-containing plaques, cerebrovascular amyloid deposits, intracellular neurofibrillary tangles of abnormally phosphorylated tau protein, astrocytic gliosis, reactive microglia, and neuronal and synaptic loss (Auld et al., 2002). A vast body of experimental data has shown an involvement and interaction of these features, consistent with the complexity of underlying causative mechanisms in AD. This review emphasizes the relevance of cholinergic mechanism and Aβ in the pathogenesis of AD.

1.1.1 Cholinergic hypothesis

In AD patients, certain brain regions and neural systems are preferentially affected, including neurons in the cortex, hippocampus, amygdala anterior thalamus, basal forebrain and several brainstem monoaminergic nuclei (Sisodia and Editor, 1999). Although several neurotransmitter systems are affected in the brain of AD patients, one

of the earliest pathological events is thought to be the degeneration of cholinergic neurons of the basal forebrain (BF) (Auld et al., 2002). In parallel with neuronal loss, choline acetyltransferase (ChAT) activity, high-affinity choline uptake, acetylcholinesterase (AChE) activity and the level of acetylcholine (ACh) are significantly decreased (Kar et al., 1996). The BF is comprised mainly of cholinergic neurons, although it also contains non-cholinergic neurons, primarily GABAergic. The basal forebrain cholinergic system in primates is comprised of cholinergic neurons in the medial septum (MS), nucleus of the diagonal band of Broca (DBB), and nucleus basalis of Meynert (NBM). The BF neuronal system provides the main cholinergic innervation to limbic and cortical brain structures (Auld et al., 2002). Specifically, neurons in the MS project predominantly into the hippocampus, whereas those of the vertical DBB and horizontal DBB innervate the anterior cingulate cortex and olfactory bulb (Auld et al., 2002). The degeneration of hippocampus and cortical cholinergic innervation is linked to the loss of memory and cognitive dysfunction observed in AD (Drachman and Leavitt, 1974; Teter and Ashford, 2002; Arendt, 2001).

1.1.2 β –amyloid

1.1.2.1 Synthesis and processing

β –amyloid, a 39- to 43-amino acid peptide and the major constituent of the senile plaques, is generally believed to play an important role in the progress of neurodegeneration and cognitive deficits. Aβ is derived from amyloid precursor protein (APP) by sequential proteolytic cleavages (Selkoe, 2001). The amyloidogenic fragments Aβ 1-40/42 are generated through sequential cleavage of APP by β- and γ-secretases

(Wei et al., 2002; Selkoe, 2001). Under normal conditions, cleavage by α- and γ-secretases generates non-amyloidogenic Aβ 17-40/42 peptides (Wei et al., 2002; Selkoe, 2001).

1.1.2.2 Role of amyloid in dementia—amyloid hypothesis

There is a substantial body of evidence to support the "amyloid cascade hypothesis" which considers Aβ as a causative agent of Alzheimer's pathology (Hardy and Higgins, 1992). First of all, cerebral Aβ deposition is an invariant feature of AD, and in virtually all cases the form of Aβ deposition is substantially more abundant in the limbic and association cortices of AD than age-matched control brains (Selkoe, 1994). Second, from genetic point of view, the mutations in APP are a cause of early onset familial AD which demonstrate all of the neuropathological and clinical features of AD (Mudher and Lovestone, 2002). Furthermore these mutations result in an increase of Aβ1-42 generation in *in vivo* and *in vitro* models and in fibroblasts from affected families (Mudher and Lovestone, 2002). In line with this evidence, Aβ is also neurotoxic to cultured cells and enhances tau phosphorylation under some conditions (Takashima et al., 1993; 1998). It is interesting to notice that although both intracellular and extracellular Aβ are toxic in rat, human cells are resistant to extracellular Aβ (Zhang et al., 2003).

However, concern about this hypothesis has been raised by some puzzling observations. The amount of neuritic senile plaques does not correlate well with the degree of dementia seen in AD patients (Arriagada et al., 1992), however there is a correlation between amount of the soluble form of Aβ in the brain and the severity of cognitive decline (Näslund et al., 2000). Other evidence comes from the transgenic

mouse model bearing the familial AD mutation shows an increase in Aβ1-42 levels and subsequent plaque pathology. However, most of these models do not show significant neuronal loss, little tau phosphorylation and no tangle formation (Mudher and Lovestone, 2002). Although some concerns exist, the "amyloid hypothesis" still receives intense attention on part of researchers as an important aspect of the pathogenesis for AD.

1.1.2.3 Mechanism of toxicity

Mechanisms of Aβ associated neurotoxicity are numerous and varied. Direct toxic mechanisms include free radical accumulation, altered calcium homoeostasis and an inflammatory response (Suh and Checler, 2002). Aβ may also target at some cell surface receptors and activate downstream signal transduction pathways. Aβ and its fragments have been shown to activate JNK and MAPK pathways (Wei et al., 2002; Troy et al., 2001; Morishima et al., 2001; Rapoport and Ferreira, 2000). Recently, increasing evidence suggests that apoptotic cell death is a key feature AD. As a key marker, a wide range of caspases have been implicated in regulating neuronal cell death in response to Aβ exposure *in vitro*, in animal models of neurodegenerative diseases, and in AD brains (Roth, 2001). Caspases are a family of cysteine-dependent, aspartate-specific proteases. These caspases, when activated, initiate a death program by destroying key cellular components and activate factors that mediate damage to the cells (Friedlander, 2003). Stadelmann et al. (1999) indicated that activated caspase-3, a central apoptotic effector, was abundant in neurons of AD patients but only observed with a low incidence in age-matched control. Allen et al. (2001) showed that treatment of $A\beta_{25-35}$ caused apoptosis of cerebellar granule cells and cortical neuronal cultures associated with increased activity of caspases-2, -3 and –6.

1.1.2.4 Aβ conformations and toxicity

Since Hardy and Higgins (1992) formalized "Amyloid Cascade hypothesis", investigators have been focusing on the fibrillar form of Aβ and regarded Aβ aggregation and deposition as a causative agent of Alzheimer's pathology. There are abundant studies to support this idea including, for example, the amyloid deposition as a hallmark of AD and neurotoxicity of fibrillar Aβ to cultured neurons (Rapoport and Ferreira, 2000).

However, the emerging view and one that is held now is that oligomeric Aβ and protofibrils have potent neurotoxicity, whereas fibrils may be end stages of the neurotoxic activity of oligomers and perhaps, not the most relevant toxin to AD (Klein et al., 2001; Kirkitadze et al., 2002; Walsh et al., 2002). With this currently held view on the toxic species of Aβ, the concerns about the weak correlation between the fibrillar Aβ load and AD severity could be explained. And thus, the focus of therapeutic strategies for AD may be shifted from fibril elimination to new goals.

1.1.3 Interaction between β –amyloid and basal forebrain cholinergic system

Recently abundant data are showing that Aβ and cholinergic systems reciprocally affect each other. Aβ could interfere with normal cholinergic transmission by inhibiting high-affinity choline uptake and ACh release in rat hippocampal slices (Kar et al., 1998). There is also evidence showing that Aβ modulate muscarinic and nicotinic acetylcholine receptors (m/nAChR) and affects downstream cascade (Kelly et al., 1996; Fu and Jhamandas, 2003; Pettit et al., 2001). On the other hand, Nitsch et al. (1992) reported that activation of mAChR could also stimulate the release of soluble APP. This indicates

Aβ can modulate cholinergic function but also suggest that Aβ processing and synthesis may be regulated by activation of cholinergic receptors.

1.2 Galanin (GAL)

1.2.1 Molecular structure of GAL

GAL, a 29 amino acid peptide, was first isolated from porcine small intestine and named for its amino-terminal glycine and amidated carboxylerminal alanine residues by Tatemoto and co-workers in 1983. NMR studies suggest that GAL adopts a predominatly α-helical structure in solution (Wennerberg et al., 1990). GAL is derived from a 123- or 124- amino acid peptide precursor named prepro-GAL. The precursor is comprised of a leader sequence, a single GAL sequence and a GAL message-associated peptide sequence of as yet unknown function (Rökaeus and Brownsterin, 1986; Kaplan et al., 1988). GAL has also been identified in other species, i.e., rat and human (Counts et al., 2001). Amongst the species, GAL is conserved in N-terminal (1-15aa) domain and different in C-terminal portion. The N-terminus has a high binding affinity to GAL receptors and C-terminal portion functions to protect the N-terminal portion from proteolytic attack (Counts et al., 2001; Fisone et al., 1989).

1.2.2 Central and peripheral distribution of GAL

Since first isolated, GAL immunoreactive profiles have been described throughout mammalian CNS and PNS in different species.

1.2.2.1 In the CNS

Melander and co-workers described the distribution of galanin-like immunoreactivity in the rat central nervous system (Melander et al., 1986). These investigators detected high concentration of galanin-immunoreactivity (GAL-IR) in hypothalamus, medulla oblongata, and spinal cord. Less extesive immunoreactivity was detected in the telecephalon, thalamus, mesencephalon, and pons, while virtually no GAL-positive structures were seen in the olfactory bulb and cerebellum.

Septum

Several groups of GAL-IR cell bodies and numerous GAL-positive fibers were observed in the septal region. Melander and co-workers detected a large group of GAL-IR medium-sized bipolar somata in MS. The second large group of GAL-IR somata was found in the ventral nucleus of DBB. GAL-positive cell bodies were also observed in dorsal to the medial border of the olfactory tubercles, extending caudally into the horizontal nucleus of DBB.

Medium density of GAL-IR fibers was observed in the lateral anterior septal nuclei, extending into both the dorsal and ventral portion of the vertical limb of DBB. GAL-positive fiber network was also detected in the intermediate and ventral lateral septal nuclei and some other nucleus in septum.

Interestingly, the distribution of GAL in the BF differs among rodents, monkeys, and humans (Mufson et al., 1998; Counts et al., 2001). In the rat, GAL co-localizes with cholinergic neurons only within the septal diagonal band but to a small degree (Melander et al., 1985; Miller et al., 1998). In the monkey, virtually all the cholinergic BF neurons contain GAL-like immunoreactivity and GAL mRNA (Mufson et al., 1998; Kordower

and Mufson, 1990), whereas in human neither the mRNA nor the peptide is present in cholinergic BF neurons (Mufson et al., 1998; Walker et al., 1991; Kordower and Mufson, 1990). However, in human a small population of non-cholinergic GALergic interneurons have been reported in the BF (Mufson et al., 1998; Counts et al., 2001).

Cortex and hippocampus

GAL-positive cell bodies were weakly labeled in the cingular cortex. GAL-IR fibers were detected throughout the cerebral cortex with at least two different morphological types. There were also two types of GAL-positive soma observed in hippocampal formation. A low-to-medium-density GAL-IR fibers were detected in subiculum. Low-density fibers were seen in stratum radiatum, dentate gyrus, extending into the stratum granulare, lateral to the hippocampal fissure, and in the ventral pole of stratum oriens.

1.2.2.2 In the PNS

GAL occurs in the respiratory tract, gastrointestinal tract and urogenital tract (Ruczynski et al., 2002;Melander et al., 1985; Cheung et al., 1985). In the nasal mucosa, brachea, bronchus, and major airways of mammalian (pig, dog, guinea pig), GAL-IR fibers were detected in smooth muscle, around seromucous glands and in the adventitia of blood vessels (Cheung et al., 1985). GAL-IR cell bodies and fibers were detected in gastric antrum, ileum, colon, as well as submucous and myenteric plexus (Melander et al., 1985; Wang et al., 1995). GAL-IR nerves were also discovered in iris and facial nerve (Stromberg et al., 1987).

1.2.3 Receptors

Three main G-protein-coupled receptor subtypes have been cloned, termed galanin receptor (GALR) 1, GALR2 and GALR3. GAL receptors share a low degree of homology. For example, hGALR1 shares 42% amino acid identity with hGALR2 and 38% with hGALR3 (Branchek et al., 2000).

1.2.3.1 GALR1

GALR1 cDNA was first isolated from a human bowes melanoma cell line (Habert-Ortoli et al., 1994). GALR1 mRNA is quite consistent with the distribution of GAL peptide expression and GAL binding sites. In rat nervous system, the highest level of GALR1 mRNA was observed in hypothalamus (supraoptic nucleus), amygdala, ventral hippocampus, thalamus, brain stem (medulla oblogata, locus ceruleus, and lateral parabrachial nucleus), and spinal cord (dorsal horn) (Branchek et al., 1998). Activation of the cloned GALR1 reduced the forskolin-stimulated increase of cAMP (Habert-Ortoli et al., 1994). GALR1 also opens G-protein-coupled, inwardly rectifying K^+ channels, and stimulates MAPK pathway in a manner that is sensitive to pertussis toxin, indicating that it couples to $G_{i/o}$-type G protein (Branchek et al., 2000; Wang et al., 1998).

1.2.3.2 GALR2

GALR2 is widely distributed in the CNS and PNS tissues. In the CNS, the highest level of GALR2 was found in the hypothalamus, hippocampus, amygdala and pyriform cortex as well as in the dentate gyrus, mammillary nuclei and cerebellar cortex (Branchek et al., 2000). Peripheral tissues where GALR2 were detected include vas deferens, prostate, uterus, ovary, stomach, large intestine, dorsal root ganglia and pancreas-derived cells (Branchek et al., 2000). Activation of GALR2 receptors with

GAL and analogues increased inositol phospholipid turnover and intracellular calcium levels in stably transfected CHO (Chinese hamster ovary) cells and generated calcium-activated chloride currents in Xenopus oocytes, suggesting the GALR2 is primarily coupled to the activation of phospholipase C (Smith et al., 1997). GALR2 is also involved in regulating forskolin-stimulated cAMP production and mediating pertussis toxin-sensitive MAPK activity (2-3 fold) (Wang et al., 1998). The signaling GALR2 activates is consistent with the activation of G_o, G_q/G11, and G_i.

1.2.3.3 GALR3

The cloning of the third GAL receptor cDNA was described by two groups (Wang et al., 1997; Smith et al., 1998). Different from GALR1 and GALR2, GALR3 has a restricted distribution in rat CNS. By northern blot analysis, Wang and co-workers only detected GALR3 mRNA in heart, spleen and testis, but not in the brain (Wang et al., 1997). Smith and co-workers reported that by the more sensitive method of solution hybridization/RPA, the GALR3 mRNA was found with the highest level in rat hypothalamus and pituitary gland and lower level in the olfactory bulb, cerebral cortex, medulla oblongata, caudate puteman, cerebellum, and spinal cord (Smith et al., 1998). They also observed low level of GALR3 mRNA expression in peripheral tissues including the liver, kidney, stomach, testicle, and adrenal cortex. In transfected Xenopus oocytes cells, GALR3 stimulates a pertussis-toxin-sensitive activation of an inward K^+ current indicating it is coupled to $G_{i/o}$-type G proteins (Branchek et al., 2000).

1.2.3.4 GALR distribution in BF

Northern blot, in situ hybridazaion and solution hybridization/RNase protection analyses demonstrate that GALR1 mRNA is relatively abundant in the BF (Counts et al.,

2001). GALR2 mRNA was also detected to co-localize with GALR1 in BF. Whether GALR3 is present in BF is yet to be determined. According to saturation experiment and the comparative radioligand GAL binding studies in human BF, it is suggested that radioligands were interacting with different GALRs within the basal forebrain and that two receptor subtypes, i.e., GALR1 and GALR2 exist in approximately the same concentration (Mufson et al., 1998). Considerable research still needs to be performed to gain an understanding of the regulation of cholinergic system by GALRs in the BF and their relationship to AD.

1.2.4 Physiological functions

Consistent with the wide spread distribution of GAL and its receptors in CNS and PNS, GAL also plays an important role in diverse physiological functions, such as cognition, nociception, feeding and endocrine activities. GAL inhibits acetylchoine release in rat hippocampus and cortex (Fisone et al., 1987; Wang et al., 1999), and impairs performance on rodent memory tasks (McDonald et al., 1998), suggesting GAL over-expression is involved in cognitive function. GAL is also involved in neuroendocrine functions. GAL was found to increase the plasma glucose level in the dog when the peptide was first described (Tatemoto et al., 1983). Later, several groups discovered that GAL could inhibit insulin release stimulated by glucose, both *in vivo* and *in vitro* (Sharp et al., 1989; Yoshimura et al., 1989). In CNS, GAL is present in anterior pituitary and regulates hormone secretion of gonadotropin, prolactin and growth hormone (Merchenthaler et al., 1993). As an active hormone in hypothalamus, GAL also plays a role in central control of feeding and body weight (Gundlach, 2002). GAL is present in

sensory and spinal dorsal horn neurons suggesting it modulates on nociceptive input at the spinal level (Xu et al., 2000). After peripheral nerve injury and inflammation, which are associated with chronic pain, GAL and GALR2 levels are significantly increased (Zhang et al., 1998). It is suggested that the increased GAL may produce antinociception on account of its inhibitory effects (Xu et al., 2000). Wynick and co-workers pointed out however that the rising levels of GAL observed under these conditions may also contribute to the initiation and maintenance of axonal regeneration in the injured neurons, leading to functional recovery and restoration of function (Mahoney et al., 2003). They further indicated GALR2 might act as a pivotal factor in this process. In GAL knockout model, these investigators also observed fewer sensory neurons and their capacity to regenerate were reduced (Holmes et al., 2000).

1.3 GAL and Alzheimer's disease

1.3.1 GAL plasticity in AD

AD is not only characterized by the cholinergic neuronal degeneration in BF, but also associated with deficits in other neurotransmitters and neuropeptides. Beal and co-workers (1990) reported in the NBM of AD patients, there were no significant changes in glutamate (Glu), aspartate, taurine and GABA levels, but a significant reduction in 5-HT and ChAT activity. Surprisingly, they found GAL immunoreactivity was dramatically increased by 98% (Beal et al., 1990). Earlier, Chan-Palay (1988) first described that in AD patients, GAL positive neurons in the brain were more frequently and intensely labeled than in controls. Recently, Mufson's group determined via a two-site ELISA assay that GAL concentrations in the nucleus basalis of AD patients were increased

~three fold compared to age-matched controls (Counts et al., 2003). They also found the increases in GAL binding sites in nucleus basalis occurred at a late stage of AD, not in early AD cases (Mufson et al., 2000). GAL binding sites were also observed to increase in hippocampal subfield in AD patients (Rodríguez-Pzertas et al., 1997).

1.3.2 GAL hypertrophy and hyperinnervation of cholinergic neurons in AD

Chan-Palay (1988) was also the first to show that GAL immunoreactive axons and dendrites innervate cholinergic BF neurons in the normal human brain. She described that the fine nets of GAL-IR axons and terminals enwrap the perikarya of Ch4 (NBM) neurons, particularly the cholinergic (ChAT positive) neurons and that GAL fibers also climb along the primary dendrites of these cells. This suggests that GAL in local circuit interneurons may provide a significant modulation or control of the cholinergic neurons and their function within the BF (Chan-Palay, 1988). Later, Chan-Palay demonstrated that in AD patients, GAL-IR fibers hypertrophy and are thicker than normal, with obviously enlarged varicosities wrapping around cholinergic perikarya or dendrites (Chan-Palay, 1988). Mufson and co-workers (1993) observed similar hypertrophy of GAL-IR fibers and hyperinnervation of cholinergic neurons by GAL-IR fibers in vertical limb of the diagonal band nucleus (Ch2). They also observed galaninergic hypertrophy and hyperinnervation of cholinergic BF neurons of AD patients by confocal microscopic analysis (Bowser et al., 1997). They demonstrated slender, beaded GAL-IR fibers in direct apposition to cholinergic BF soma (Bowser et al., 1997).

1.3.3 Controversies on the role of GAL in AD

1.3.3.1 GAL as a detrimental factor

The mechanism responsible for GAL over-expression in AD brains remains unclear. However, the over-expression of GAL in the BF suggests that this peptide may modulate cholinergic function in AD either positively or negatively. For some time it has been suggested that GAL over-expression may exacerbate cholinergic dysfunction and impair cognition. A body of data from *in vivo*, *in vitro* experiments and transgenic models has been invoked to support this hypothesis.

GAL acts as an inhibitory transmitter in a number of nuclei in CNS. GAL inhibits ACh release in the ventral, but not in the dorsal, rat hippocampus, *in vivo* and *in vitro* (Fisone et al., 1987). Wang and co-workers (1999) observed same inhibition of acetylcholine release induced by GAL in rat cerebral cortex. Electrophysiological studies show GAL to inhibit the slow cholinergic excitatory post-synaptic potential (EPSP) in CA1 pyramidal neurons from rat ventral hippocampus and to inhibit long-term potentiation in guinea-pig hippocampal slices (Dutar et al., 1989; Sakurai et al., 1996). Electrophysiological data also showed that GAL decreases the firing rate in slices from rat locus coeruleus neurons and hypothalamic magnocellular neurosecretory cells (Seutin et al., 1989; Papas and Bourque, 1997). It has been presumed that GAL may have similar inhibitory actions in BF to cause a reduction in cholinergic tone and hence an impairment of cognition.

In behavioral studies, GAL inhibits performance in a variety of rodent learning and memory tasks. Central administration of GAL impaired performances on delayed non-matching to position in rats; T-maze delayed alternation in rats; starburst radial maze

in rats; passive avoidance in mice; and the Morris water task in rats (McDonald et al., 1998). Wrenn and Crawley (2001) inferred that the mechanisms underlying these memory deficits could be explained by an inhibition of neuronal firing rate and transmitter release caused by GAL, or an activation of signal transduction pathways following the GAL receptor binding activity. Several putative GALR antagonists have been developed designated M15, M30 and M40. The inhibitory actions of GAL on memory tasks are blocked by the peptidergic antagonist M40 (McDonald et al., 1998). Co-administration of GAL antagonist M40 with a muscarinic M_1 agonist improves delayed non-matching to position choice accuracy in rats with cholinergic lesions (McDonald et al., 1998). These results are in accordance with the inhibitory effect of GAL in rodent memory tasks. However, there are some concerns with these behavioral studies. First, the dose of GAL used in memory tasks could be much higher than under physiological conditions. It is also possible that the memory deficits caused by the high levels of GAL in same studies are not likely to be induced by the level of GAL in AD patients. Second, the GALR antagonist, M40, has been reported to exhibit some agonist properties (Smith et al., 1997). If M40 acts as an agonist in this case, then effects of GAL and M40 in such studies would need to be re-evaluated.

In a GAL over-expressing transgenic model generated by Steiner et al. (2001), GAL expression is approximately two-fold higher in the forebrain and is also elevated in other nuclei compared with WT control (Steiner et al., 2001). In this model, the GAL transgenic mice showed performance deficits on learning and memory tasks, impaired LTP, reduced hippocampal excitability, lower evoked glutamate release, and reduced ChAT immunoreactive neurons in the horizontal DBB (Crawley et al., 2002).

Collectively, these observations have been invoked to support the hypothesis that GAL over-expression acts to inhibit neurotransmitter ACh release and impair cognition.

1.3.3.2 GAL as a neuroprotective factor

In spite of the above-mentioned studies, emerging data has also led to an alternative hypothesis that GAL may have a neuroprotective role in degeneration of cholinergic BF observed in AD patients.

In an autoradiographic study of GAL binding site in AD brain sections, Mufson and co-workers found a two-three-fold increase of [^{125}I]hGAL binding in anterior NBM in late stage AD (Mufson et al., 2000). This region is relatively spared in AD (Mufson et al., 1989). However in the posterior portion of NBM, which undergoes severe degeneration in AD, [^{125}I]hGAL binding densities were not significantly different in early or late AD compared to age-matched controls (Mufson et al., 2000). In a rat model with chemical lesion of the BF, no correlation between the GAL hypertrophy and cholinergic cell loss was observed (De Lacalle et al., 1997). These findings suggest that GAL upregulation might serve not only as a response to cholinergic degeneration, but exert a neuroprotective effect. Thus, an increase in GAL binding sites may play a role in the preservation of cholinergic BF neurons.

Another piece of evidence for the alternate GAL hypothesis comes from nerve growth factor (NGF) study by Planas et al. (1997). NGF, a neurotrophin factor for cholinergic BF neurons, will increase ACh synthesis and release when administered centrally in rats (Rylett et al., 1993). Using in situ hybridization and quantitative autoradiography, they reported that NGF strongly up-regulated GAL gene expression in

cholinergic BF as well as ChAT gene in the rats following chronic intracerebroventricular NGF infusion (Planas et al., 1997). In keeping with the neurotrophic effects of NGF in the CNS, NGF induction of GAL gene expression may be an important component of the trophic actions of NGF on neuronal function, hence increasing cholinergic tone in BF.

A recent electrophysiology study from our lab demonstrated novel excitatory actions of GAL on cholinergic BF neurons, different from its traditional inhibitory role reported elsewhere in the CNS (Jhamandas et al., 2002). In this study, application of GAL to acutely dissociated rat BF DBB neurons caused a decrease in whole-cell voltage-activated currents in a majority of cells. By the use of single-cell RT-PCR technique, this effect was deemed to be specific to cholinergic, but not GABAergic neurons. Under current clamp conditions, GAL depolarized cholinergic neurons, an effect that was mimicked by even nanomolar co-application of the putative GALR antagonist M15 (galantide). This observation suggests that the GAL excitatory actions may serve a compensatory role by augmenting the ACh release and delay the progression of AD pathology, particularly when the levels of this peptide are upregulated as reported in AD brains (Jhamandas et al., 2002).

The "neuroprotective" GAL hypothesis is also supported by a transgenic mouse model carrying a loss-of-function mutation in GAL gene generated by Wynick and co-workers (O'Meara et al., 2000). In this model, they surprisingly observed the loss of one-third of cholinergic neurons in MS and vertical limb DBB of BF; a two-fold increase in the number of apoptotic cells in the forebrain at postnatal day seven; deficits in stimulated ACh release, performance in the Morris water maze, and induction of LTP in the CA1 region of hippocampus (O'Meara et al., 2000). These observations indicate that

GAL might act as a trophic factor in regulating the survival, development and function of cholinergic BF neurons.

Additional *in vivo* and *in vitro* studies are also consistent with the effect of GAL as a protective neuromodulator. In a rat traumatic brain injury model, GAL pretreated rats produced less magnitude of deficits compared to CSF treated ones on beam-balance, beam-walking, and other performances (Liu et al., 1994). In the periphery, Wynick and co-workers demonstrated that the rising levels of GAL after nerve injury may contribute to the initiation and maintenance of axonal regeneration in the injured neurons, leading to functional recovery and restoration of function (Mahoney et al., 2003). In CNS, neuronal hipocampal primary cultures from GAL knockout mice are more sensitive to kainate and staurosporine toxicity in comparison with wild type (WT) cultures, while cultures from GAL over-expressing models showed even less cell death compared to WT (Elliott-Hunt et al., 2004). In WT cultures, GAL significantly reduced staurosporine-induced and Glu-induced cell death, which could also be mimicked by GALR 2 specific angonist AR-M1896 (Elliott-Hunt et al., 2004).

Based on these collective data, we hypothesize that GAL might be neuroprotective in rat cholinergic BF for the following reasons. First of all, we observed excitatory effect of GAL on cholinergic BF neurons in contrast to the inhibitory effects for this peptide that have been reported in hippocampus and cortex. It is possible that these differences, i.e., excitatory versus inhibitory effects, may relate to the specific GALR that is activated. Second, in primary hippocampal culture, GAL acts as a neuroprotective factor against staurosporine and Glu toxicity suggesting that GAL may also work by the same mechanism in BF. Finally, the reduction of cholinergic activity

and deficits in memory tasks observed in GAL knock out mice indicate that GAL may work as a trophic factor in regulating the survival, development and function of cholinergic BF neurons.

The goal of this study is to exam the effect of GAL against Aβ toxicity in rat BF neuronal primary cultures. We used our primary cultures of cholinergic BF neurons and performed apoptotic cell death assays to investigate the cell viability after GAL and Aβ treatment. We also tested the effect of a GALR agonist specific for the GALR2. Finally we investigated the neuroprotective effect of GAL against Aβ-induced activation of caspase 3, an executioner caspase.

Chapter 2

Materials and Methods

2.1 Primary cell cultures

Primary rat basal forebrains were cultured from 17-d to 18-d-old fetuses of time-dependent pregnant Sprague Dawley rats from Charles River Canada. Procedures for cell cultures have been previously described (Mennicken and Quirion, 1997). Septal regions containing the cholinergic neurons were dissected in Hanks balanced salt solution (Invitrogen, Burlington, Ontario, Canada) supplemented with 15 mM HEPES, 10U/ml penicillin, and 10mg/ml streptomycin. Tissue pieces were then digested with 0.05% tripsin at 37℃ (15 min). Digestion was terminated by addition of fetal bovine serum. The cellular suspension was then completed by repeated aspirations through a fire-polished small-bore Pasteur pipette. Following centrifugation for 10 min (800g), cells were plated on a 96 well plate (5×10^5 per well) for biochemical studies or on 12-mm-diameter coverslips (coated with 10mg/ml poly-D-lysine) for anatomical studies. The growth media consists of neurobasal media, 15mM HEPES, 10U/ml penicillin, 10mg/ml streptomycin and 2mM sodium pyruvate, supplemented with N_2 or B_{27}. The cultures were grown at 37℃ (5% CO_2) in a 5% humidified atmosphere.

2.2 Acetylcholinesterase histochemistry

Enzymatic staining of AChE was performed according to the procedures recommended by Tago et al. (1896), slightly modified in our laboratory for primary cultures. 6-8 Day cultured cells on coverslips were fixed for 45 minutes in 4% paraformaldehyde and then washed three times with 0.1M Maleate buffer (pH 6.0), then incubated for 2 hours in the dark in a fresh solution consisting of 300μM $CuSO_4$ 500μM sodium citrate, 50μM $K_3Fe(CN)_6$, 30μM acetylthiocholine iodide in 0.1M maleate buffer. Cultures were then rinsed with PBS for 5 times, incubated in intensification solution consisting of 0.04% 3,3' diaminobenzidine tetrahydrochloride, 0.3% nickel ammonium sulfate and 0.003% H_2O_2 in 0.1M PBS for 15-30 minutes. Finally, the coverslips were dried, dehydrated, and mounted on glass slides.

2.3 Immunocytochemistry

Cultures on coverlips were fixed for 45 minutes in 4% paraformaldehyde followed by three rinses with phosphate buffered saline (PBS; pH 7.2-7.4). Primary antibodies to neuron-specific enolase (NSE; 1:1000; made in mouse), to ChAT (1:200; made in rabbit, gift from Dr. Kar) or to vesicular acetycholine transporter protein (VChAT, 1:500; made in rabbit; Sigma) were diluted in PBS with 0.3% Triton X-100 and added to the culture for overnight incubation at 4℃. Cultures were washed with PBS for 3 times the next day and were incubated in secondary antibody (anti-mouse for NSE staining; goat anti-rabbit IgG for ChAT and VChAT staining, Vector Laboratories, Burlingame, CA). Finally culture was stained by avidin-biotin-peroxidase (ABC) method (1:600; Vector Laboratories). ABC method is an immunoenzymatic technique, which

involves the application of biotinylated secondary antibody, followed by the addition of avidin-biotin-peroxidase complex, thus giving an amplified result (Hsu et al., 1981). After that the culture was exposed to 0.04% DAB in PBS with 0.003% H_2O_2 before the coverslips were dried, dehydrated, and mounted on glass slides. Cultured cells were examined under Zeiss microscope system. Three to four fields were randomly chosen, averaged and expressed as percent cell positive.

2.4 Treatment and cell death assays

Freshly solubilized $A\beta_{1-42}$, $A\beta_{25-35}$, their respective inverse (biologically inactive) forms, $A\beta_{42-1}$, $A\beta_{35-25}$, Glu, GAL peptide (rat; Aβ and GAL are from American peptide, Sunnyvale, CA) and GAL receptor 2 agonist AR-M1896 (gift from Dr. Schmidt, AstraZeneca, Montreal) were prepared to treat the cultured cells for experiments. To determine the dose response of GAL peptide, cultured neurons were treated with different concentrations (0.1-5.0 μM) of the peptides. In each experiment and in subsequent experiments described below, each row of seven wells (of a 96 well plate) received the same treatment, and each experiment was repeated a minimum of three times. To evaluate the neuroprotective effects of GAL and AR-M1896 against Aβ toxicity, cultures were exposed to GAL or AR-M1896 (0.1 μM and 1.0 μM) for 24 hr and then 20 μM $A\beta_{1-42}$ or $A\beta_{25-35}$ was applied for 48 hr. Dose of Aβ used was based on pre-studies from our lab (Jhamandas and Mactavish, 2004), and work of others (Kar, et al.). To further evaluate the neuroprotective effects of GAL against Glu-induced excitotoxicity, cultures were exposed to GAL (0.1 μM and 1.0 μM) for 24 hr then exposed to Glu together with GAL for 48 hr. Dose of Glu used was based on Glu dose response on BF neuronal

cultures (data not shown). Cells in adjacent rows of wells received applications of either 20 μM $A\beta_{1-42}$ or $A\beta_{25-35}$ without GAL or AR-M1896 pretreatment. After 48 hr, the control and treated cultures were processed for (1) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) to measure neuronal survival. MTT is a commonly used assay for cell proliferation and cytotoxicity by measuring mitochondrial function based on the reduction of the tetrazolim salt (Berridge and Tan, 1993). (2) Live-dead assay (Molecular Probes) was applied on cultured cells on coverslip, which were treated in the same manner with Aβ peptide and GAL (or AR-M1896). The live-dead viability-cytotoxicity assay is a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells with two probes which measure two recognized parameters of cell viability—plasma membrane integrity and intracellular esterase activity (Papadopoulos et al., 1994). Quantification was assessed in the same system and same manner as in immunohistochemistry study.

2.5 Double immunofluorscent staining

For double staining primary cultures with anti-cleaved caspase-3 (made in rabbit; Cell Signaling) and anti-VChAT antibodies, cells were applied to glass coverslips and treated in the same manner with Aβ peptide and GAL. Cells were fixed for 45 minutes with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in Tris buffered saline (TBS, pH 7.4). After blocking non-specific protein with 1% Albumin bovine serum (BSA, Sigma) in TBS with 0.2% Triton, cells were then incubated with anti-cleaved caspase-3 antibody (1:250) over night at 4℃. On the following day, cells were rinsed for 3 times and incubated with secondary antibody Alexa Fluor 488 chicken anti

rabbit IgG (Green; 1:300; Molecular Probes) for 1.5 hours. Cells were rinsed again and incubated with anti VChAT antibody (1: 1000) over night 4℃. On the third day, cells were rinsed and incubated with biotinylated anti-rabbit IgG (1:250; Vector Laboratories) for 1.5 hours followed by the incubation with Steptavidin Alex Fluor 546 conjugate (Red; 1: 300; Molecular Probes) for 1.5 hours. Finally, coverslips were rinsed, dehydrated, and mounted on glass slides. Quantification was assessed in the same system and same manner as in immunohistochemistry study.

2.6 Immunoblotting

Western blotting was performed as described previously (Jhamandas and MacTavish, 2004). Briefly, samples of control cells and treated groups of cultured cells (treated with $A\beta_{1-42}$, and GAL pretreatment followed by $A\beta_{1-42}$) with equal amounts of protein were separated by 4-20% polyacrylamide gel electrophoresis, and the resolved proteins were transferred onto nitrocellulose membranes and probed with anti-cleaved caspase-3 (made in rabbit; Cell Signaling).

2.7 Statistics

Data are presented as mean ± SEM and were analyzed using *student t* test with the level of significance set at $p < 0.05$.

Chapter 3

Results

3.1 Characterization of BF primary cultures

We utilized a combination of histochemical and immunocytochemical methods to characterize the cholinergic cell phenotype in primary neuronal cultures. An average of 76.4±0.5% of the cells is positive for NSE staining, indicating that a significant portion of cells in our primary cultures are neuronal (Figure 1). The percentages of cells positive for AChE staining, VChAT and ChAT immunochemical staining were 78.1±2.9%, 81.3±1.4% and 77.0% (one experiment), respectively (Figure 1). This indicates that using a variety of different staining methodologies, the primary neuronal cultures we used consist mainly of cholinergic neurons.

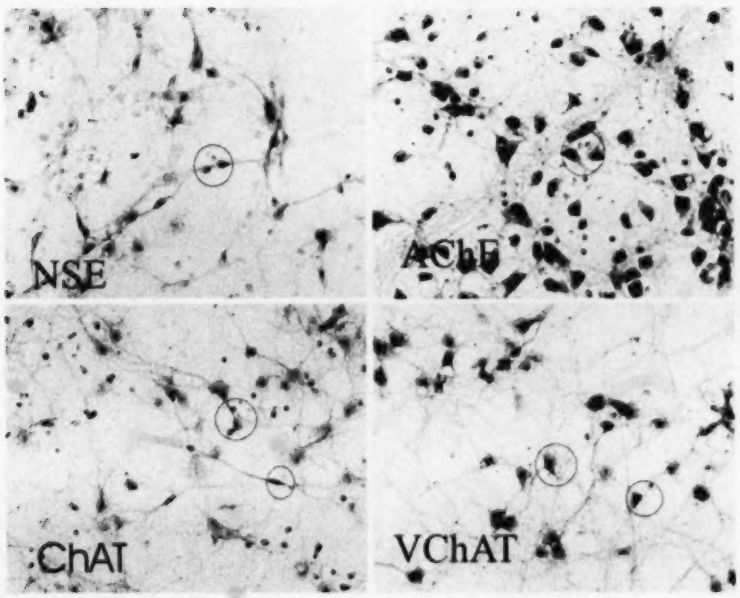


Figure 1: Histochemistry and immunocytochemistry for rat primary neuronal septal cultures. The primary cultures contain 75-80% cholinergic neurons. Circled cells are positive for neuronal or cholinergic markers.

3.2 GAL effects on BF neurons

In an initial set of experiments, the effects of GAL (dose range 0.1μM-5.0μM) on primary BF cultures were assessed. GAL alone was applied for 48 hrs and cell viability was measured using MTT assay. We observed a significant decrease in cell survival for the cells treated by GAL at a dose of 5.0μM (76.8±3.2% of neurons survived compared to controls Figure 2, $p<0.05$). No significant decrease of cell viability was observed in cells treat with GAL 0.1μM-1.0μM (Figure 2). We chose 0.1μM and 1.0μM for our further studies on the neuroprotective effects of GAL against Aβ toxicity.

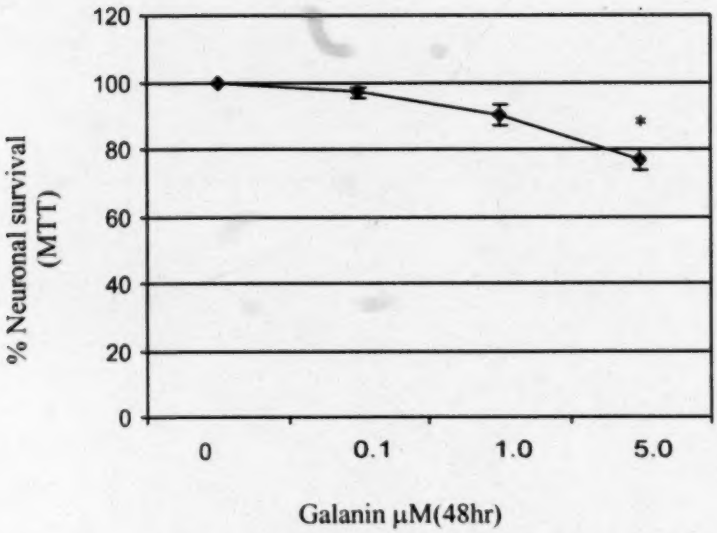
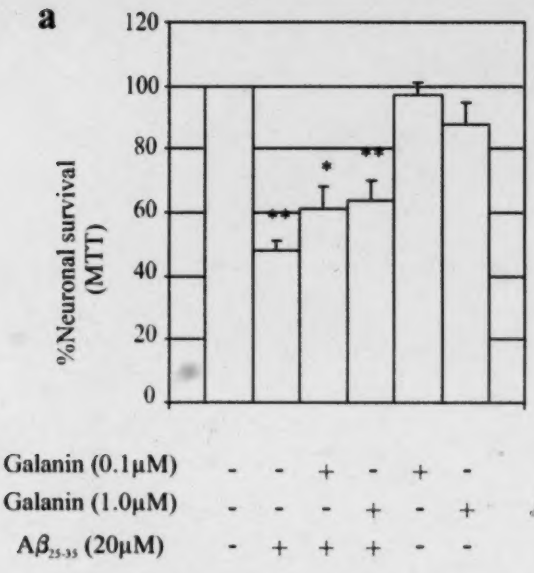


Figure 2: Galanin dose response on primary culture. Different doses of galanin (0.1-5.0μM) were applied for 48 hrs before the cell viability was tested by MTT assay.

3.3 Effect of GAL against Aβ toxicity on BF primary culture using MTT and live-dead assays

We have already demonstrated that Aβ ($A\beta_{25-35}$ and $A\beta_{1-42}$) is toxic to primary BF neurons in a concentration- and time-dependent manner (Jhamandas and MacTavish, 2004). We chose Aβ ($A\beta_{25-35}$ and $A\beta_{1-42}$) dose of 20μM as the concentration to induce cell death in our studies. Cell death was measured at 48 hrs post exposure to Aβ. Aβ, either $A\beta_{25-35}$ or $A\beta_{1-42}$ decreased cell viability by 50-60% as measured in MTT assay. Applications of inverse-sequence Aβ peptides ($A\beta_{35-25}$ and $A\beta_{42-1}$) were used as control and were not neurotoxic to BF cultures. Pretreatment of the BF primary cultures for 24 hours with GAL resulted in a significant improvement by 15-20% in neuronal survival ($p<0.05$, Figure 3). The GAL-induced attenuation of neurotoxicity evoked by $A\beta_{25-35}$ and $A\beta_{1-42}$ was also observed when it was co-applied with Aβ, although not to the same degree as pretreatment with the drug (data not shown).

The neuroprotective effects of GAL on Aβ toxicity were also observed visually by phase-contrast microscopy of cultures following GAL and Aβ treatments (Figure 4). Control cells had large cell bodies, clear cell membrane, long dendrites and prominent axons (Figure 4a). Aβ treated cells displayed less healthy morphological features with smaller cell bodies and a lesser number of axons and dendrites (Figure 4b). GAL pretreated cells showed a morphologic enhancement of cells compared to those treated with Aβ alone (Figure 4c).



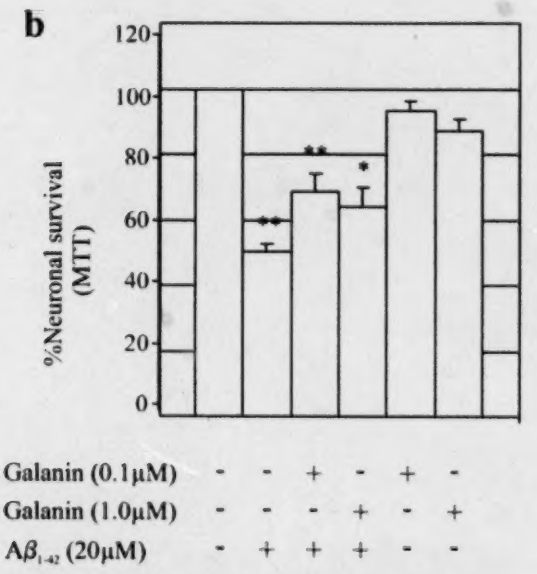


Figure 3: GAL protects BF neuronal culture against Aβ toxicity as measured by MTT assay. Histograms show that $A\beta_{25-35}$ (**a**)or $A\beta_{1-42}$ (**b**) application for 48 hr produces a significant decrease in cell viability as measured by MTT assay. Pretreatment with GAL for 24 hr significantly attenuates Aβ- induced cell death. GAL itself does not induce significant alteration in neuronal survival (*p<0.05; **p<0.01).

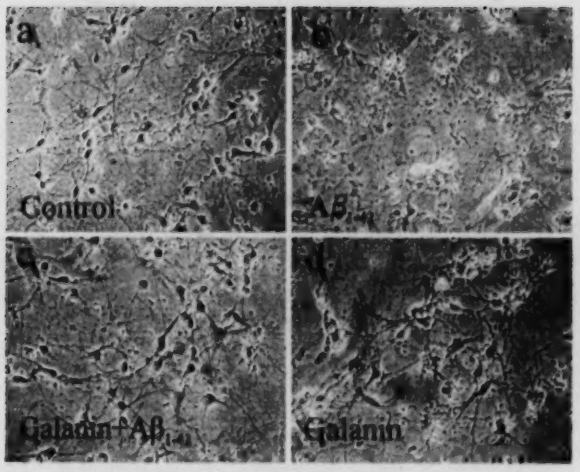


Figure 4: Phase-contrast photomicrographs of neuronal cultures treated with control media, $A\beta_{1-42}$ (20μM), GAL and Aβ, and GAL (1.0μM).

Aβ toxicity and the neuroprotective effects of GAL against Aβ toxicity were also measured using the live-dead assay (Figure 5, 6). Using the live-dead assay, the BF culture cells, after the treatment, were stained with either green fluorescence (live) or red fluorescence (dead) (Figure 5). Majority of the cells in control group are live while in the Aβ treated group, there are significantly more dead cells than live cells, which is consistent with the toxicity of Aβ towards BF cultured neurons (Figure 5a, 5b). There was an increase of live cells in GAL pretreated group compared with Aβ group (Figure5c).

The live cells and dead cells were counted in each group and statistical analysis was performed (Figure 6). GAL 0.1μM and 1.0μM pretreatment increased neuronal survival by 13%-17% compared with Aβ treated cells. These data are consistent with those obtained from study performed using MTT assay as a measure of neuronal survival (Figure 3).

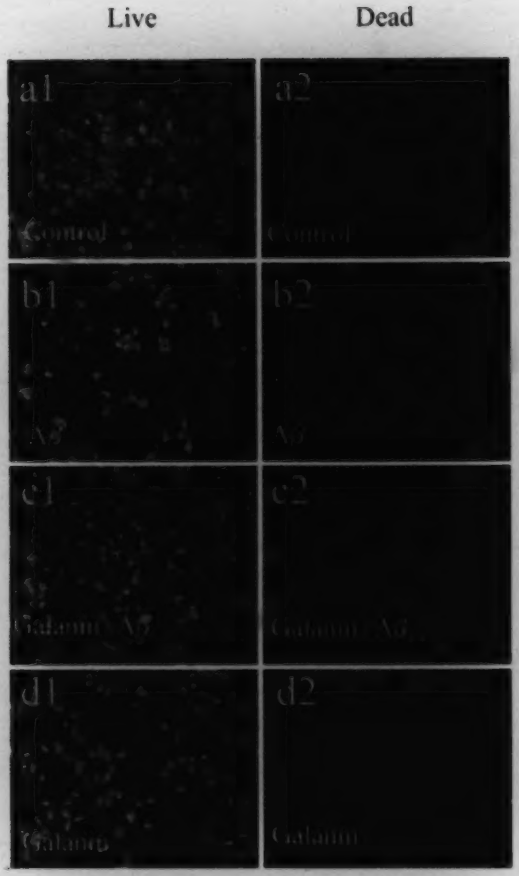


Figure 5: Live-dead assay photomicrographs of neuronal cultures treated with $A\beta_{1-42}$ and GAL. $A\beta$ induced cell death in cultured neurons (b1,b2) was attenuated by GAL (c1,c2).

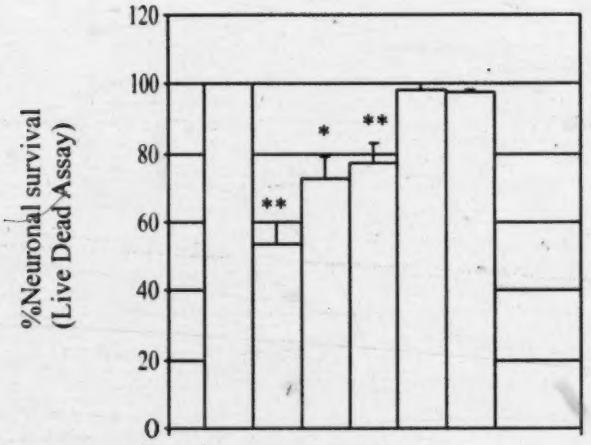


Figure 6: Live-dead assay on neuronal cultures treated with $A\beta_{1-42}$ and GAL. GAL protects BF neuronal culture against $A\beta$ toxicity . Histograms show that compared to controls, $A\beta_{1-42}$ application for 48 hr produces a significant decrease in cell viability as measured by live-dead assay. Pretreatment with GAL for 24 hr significantly attenuates $A\beta$ induced cell death. GAL itself does not induce significant alteration in neuronal survival compared to control (*$p<0.05$; **$p<0.01$).

3.4 Caspase 3 pathway is involved in Aβ induced neurotoxicity in BF neuronal culture and neuroprotective effects of GAL

Using western blotting, we examined whether GAL pre-treatment attenuated the Aβ-induced activation and cleavage of caspase 3. We observed presence of elevated products of caspase 3 in $A\beta_{1-42}$ treated cultures. In contrast, very little of the cleaved caspase 3 was detected in the GAL pretreated group (Figure 7). Then we used double immunofluorescent staining (anti-cleaved caspase-3 and anti-VChAT) to ask whether the neuroprotective effects of GAL were specifically directed towards cholinergic neurons. We observed consistent decrease in caspase 3 negative cells (cells not undergoing apoptosis) in the $A\beta_{1-42}$ treated group compared with control cultures (Figure 8, 9a). The decrease was partly reversed by GAL pretreatment (Figure 8, 9a). Using VChAT staining as a marker for cholinergic neurons, we observed a significant decrease in the percentage of cholinergic neurons in the BF neuronal cultures following Aβ treatment, which was also partially reversed by GAL pretreatment, though not significantly (Figure 8, 9b). Combining these two markers, we analyzed the caspase 3 activity in cholinergic neurons in the neuronal cultures after Aβ and GAL treatments. As expected, there was a significant decrease of the caspase 3 negative cells (cells not undergoing apoptosis) in cholinergic neurons of $A\beta_{1-42}$ treated group compared with controls (Figure 9c). This was also partially reversed by GAL pretreatment (Figure 9c), suggesting that the neuroprotective effect of GAL against Aβ toxicity were directed mostly towards cholinergic neurons. There was no significant alteration of the number of either caspase 3 positive or VChAT positive cells compared with control after the cells were treated with $A\beta_{42-1}$ or GAL alone.

Galanin (1.0μM)	-	+	+	-
$A\beta_{1-42}$ (20μM)	+	+	-	-

Figure 7: Western blotting demonstrating the activation of Caspase 3 in the BF cultures, which was inhibited by GAL pretreatment.

Caspase 3 VChAT

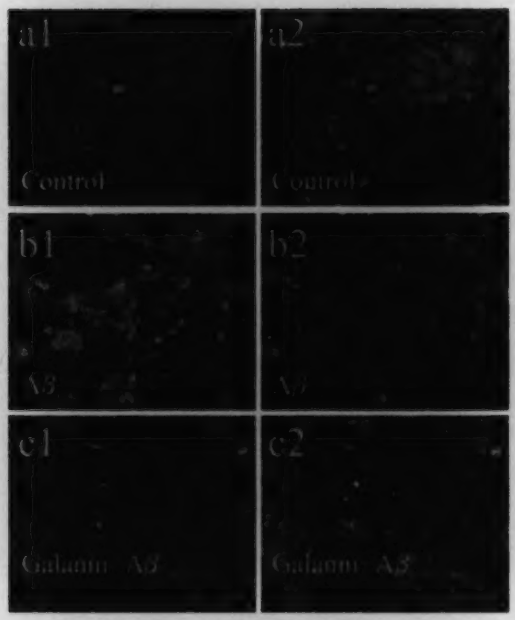
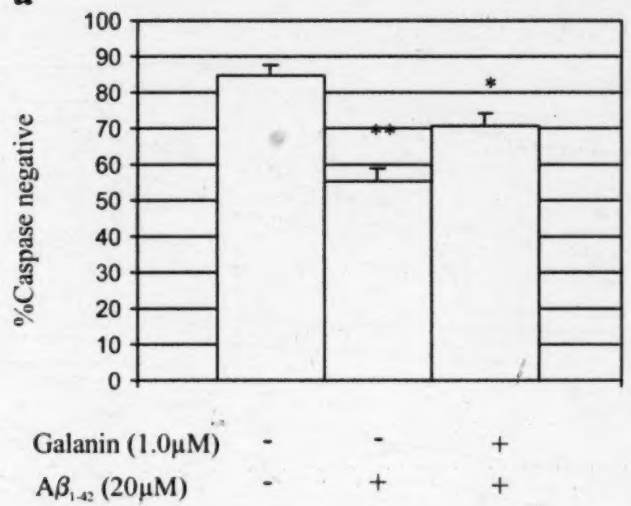
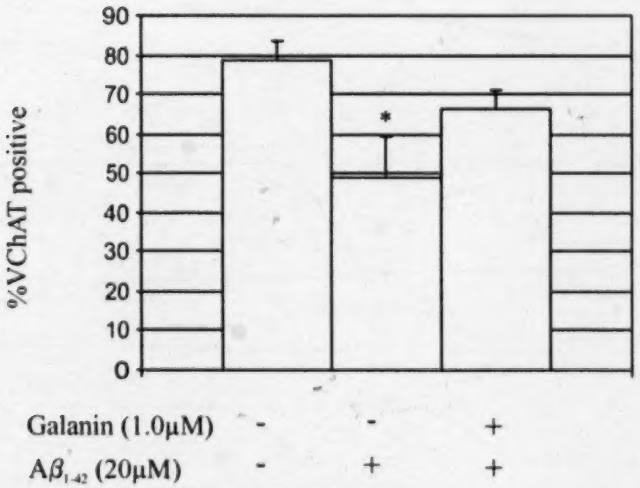


Figure 8: Aβ induced caspase 3 activity in cultured BF neurons that was inhibited by GAL pretreatment. Caspase 3 and VChAT double immunofluorescent staining photomicrographs of neuronal cultures treated with Aβ_{1-42} and GAL. For the double staining, cells expressing caspase 3 are green (Figure 7a1,b1,c1) and cells expressing VChAT are red (Figure 7a2,b2,c2).

a


%Caspase negative
100
90
80
70
60
50
40
30
20
10
0
**
*
Galanin (1.0μM) - - +
Aβ1-42 (20μM) - + +

b


%VChAT positive
90
80
70
60
50
40
30
20
10
0
*
Galanin (1.0μM) - - +
Aβ1-42 (20μM) - + +

c

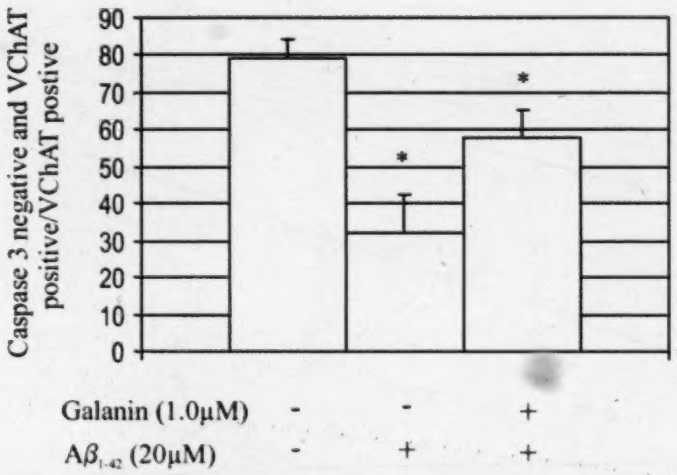


Figure 9: Histograms depicting percent of cells with immunofluorescent staining for caspase 3 and VChAT to total cells, and the percent of cells with double staining to the total cells stained for VChAT (*$p<0.05$). Aβ induced a significant increase in the percent of caspase 3 positive cells in total cells (**a**), or in total cholinergic (VChAT positive) cells (**c**). GAL pretreatment partially reversed the Aβ-induced activation of caspase 3 (**a**,**c**). Aβ also significantly decreased the percent of cholinergic (VChAT positive) cells in the cultures, however, GAL pretreatment did not significantly increase the percent of cholinergic cells compared to Aβ group (**b**).

3.5 GAL neuroprotective effects are mimicked by GALR 2 agonist AR-M1896

Similar neuroprotective effects were observed as for GAL when we used GALR 2 agonist AR-M1896 as a pretreatment prior to exposure of BF cultures to $A\beta_{1-42}$. A 15-20% increase of cell viability was observed when cells were pretreated by AR-M1896 compared with the Aβ group, and measured using live-dead assay. Surprisingly, the neuroprotective effects of AR-M1896 were more modest when cell viability was tested by MTT assay (data not shown).

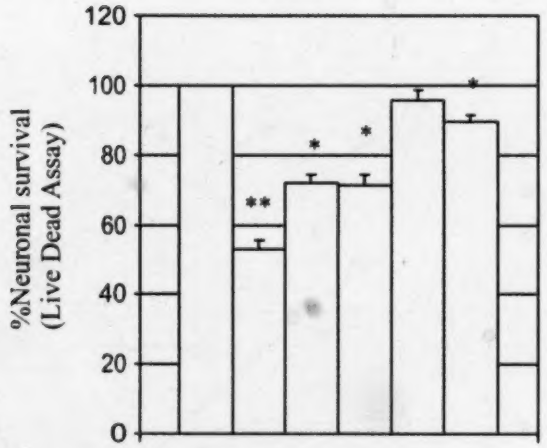


Figure 10: ARM1896 protects BF neuronal cultures against Aβ toxicity . Histograms show that Aβ_{1-42} application for 48 hr produces a significant decrease in cell viability as measured by live-dead assay. Pretreatment with ARM1896 for 24 hr significantly attenuates Aβ induced cell death. ARM1896 at dose of 0.1μM does not induce significant alteration in neuronal survival, however, ARM1896 at dose of 1.0μM significantly decreases neuronal survival by 10% (*p<0.05, **p<0.01).

3.6 GAL is neuroprotective on Glu-induced neuronal cell death

To further explore the neuroprotective effects of GAL, we also investigated the effect of GAL pretreatment on Glu-induced excitotoxicity. We observed significant increase of neuronal survival in cells pretreated by GAL than those treated by Glu alone by 10% (at GAL dose of 0.1μM, $p<0.05$) and 17% (at GAL dose of 0.01μM, $p<0.01$) (Figure 11).

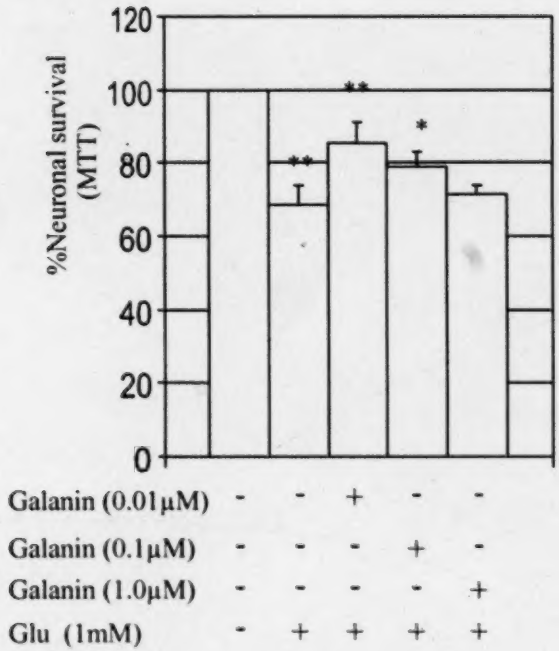


Figure 11: GAL protects BF neuronal culture against Glu excitotoxicity. Glu application for 48 hr produces a significant decrease of cell survival ($p<0.01$). GAL pretreatment significantly attenuates Glu-induced toxicity and improved cell survival by 10-20% at GAL doses of 0.1μM and 0.01μM (*$p<0.05$, **$p<0.01$).

Chapter 4

Discussion

4.1 Overview

The experimental data described in this thesis suggest an important role for GAL in the BF pathology of AD. Using BF primary neuronal culture system, we demonstrate that GAL pretreatment increases survival of cholinergic BF against Aβ induced apoptotic cell death, hence, providing evidence for the neuroprotective effects of GAL in the brain. Furthermore, we demonstrate that the neuroprotective effects of GAL occur via a blockade of caspase 3 activation evoked by Aβ. Our findings also suggest that these GAL effects on cholinergic BF neurons are likely mediated through the activation of GALR2. These data may help clarify the current controversies on the role of GAL in AD, and assist in GALR based drug development for AD patients.

4.2 GAL is neuroprotective in BF neuronal culture against Aβ toxicity

We report GAL is consistently neuroprotective against Aβ toxicity in cholinergic BF neuronal culture as judged by visual observation, MTT assay, live-dead cell assay and caspase 3 activation. MTT assay is a traditional assay for cell proliferation and cytotoxicity. This assay mainly measures mitochondrial function based on the reduction of the tetrazolium salt (Berridge and Tan, 1993). By assay of MTT, we measured a 15-

20% increase of neuronal survival by GAL pretreatment compared with Aβ group. However, when we pretreated cultures with GALR2 agonist AR-M1896, we observed only moderate improvement of neuronal survival (5-10%, data not shown) by MTT assay. In AD, one of the possible mechanisms of Aβ toxicity is the generation of free radicals and oxidative stress, which mitochondria are very sensitive to (Eckert et al., 2003). Meanwhile, there are other pathogenetic mechanisms such as disrupted calcium homoeostasis, inflammatory response and activation of intracellular apoptotic signals. Therefore, a measure of mitochondria activity is but one of neuronal indicators of cell dysfunction and death. Finally, in our septal culture model we occasionally observed some discrepancy between the integrity of cell morphology and cell survival as measured by MTT. Hence, we used a parallel live-dead assay to complement our morphological observations and MTT assay. The live-dead cell viability/cytotoxicity assay is based on the determination of live and dead cells by measuring intracellular esterase activity and plasma membrane integrity. By live-dead assay, we observed significant improvement of cell survival against Aβ toxicity by pretreatment of GAL and AR-M1896. Both the MTT and live-dead assays are a measure of cell viability. We next addressed the issue of which apoptotic pathway is involved in the neuroprotective effects of GAL. Based on previous studies identifying caspase 3 as the primary executioner caspase consequent to Aβ exposure (Allen et al., 2001), we examined activation of caspase in the context of neuroprotective effects of GAL. We used cleaved caspase 3 as an apoptotic marker in our study. As a central apoptotic effector, caspase 3 is activated and cleaved by a variety of intracellular mediators, leading to the disruption of various cellular proteins that culminate in apoptotic cell death (Chang and Yang, 2000). By use of Western blotting,

we observed a higher level of cleaved caspase 3 expression in Aβ treated cells compared with control treated cells. This increase in cleaved caspase 3 expression was reversed in GAL pretreated cells. These observations indicated that exposure to Aβ induced apoptotic cell death in the BF neuronal cultures, which was inhibited by GAL pretreatment. Next we were interested in determining whether the neuroprotective effects of GAL were directed specifically towards cholinergic neurons in the BF culture. By double immunofluorescent staining with anti-VChAT and anti-cleaved caspase 3, we confirmed our observations from Western blotting. The immunofluoresent staining consistently showed that Aβ increased cleaved caspase 3 expression which was reversed by GAL pretreatment. Secondly, we analysed chemical phenotype of the cultures after Aβ and GAL treatment and found a significant decrease of the percentage of cholinergic neurons in the BF cultures after Aβ treatment. The pretreatment with GAL increased, however not significantly, the percentage of surviving cholinergic neurons in the BF cultures compared with Aβ treated culture. Finally we combined the VChAT staining and cleaved caspase 3 staining to explore the apoptotic cell death among cholinergic neurons in the BF culture after Aβ and GAL treatment. We observed that Aβ significantly increased cleaved caspase 3 expression in cholinergic neurons in the culture, indicating that Aβ induced significant apoptotic cell death. GAL pretreatment attenuated the increase of cleaved caspase 3 expression induced by Aβ, partially rescuing the cholinergic neurons from apoptotic cell death.

It is important to highlight that in our study, GAL pretreatment partially attenuated Aβ toxicity at the doses of 0.1μM and 1.0 μM. At higher doses, an apparent toxic effect was observed when GAL is applied alone, as shown in GAL dose-response

(Figure 2). In other culture models, the inhibitory role of GAL has also been observed at high micromolar dose (Giustina et al., 1994). To our knowledge, the dose range in our study (0.1μM-1.0μM) is among the lowest used in all the GAL studies in culture models. Recently, Wynick's group also reported that at the dose of 0.1μM, GAL acts as a neuroprotective factor in hippocampal cultures (Elliott-Hunt et al., 2004). The different properties GAL displays at varying concentrations may help explain the controversies on the role of GAL in AD.

The conformations of Aβ, i.e. monomeric, oligomeric or fibrillar, is important in the context of Aβ-induced toxicity. As an emerging point of view, soluble oligomeric form of Aβ is thought to have the most potent toxic effects with the fibrillar form less toxic (Klein et al., 2001; Kirkitadze et al., 2002; Walsh et al., 2002). We presume that in our experiments, there are several of Aβ species present since although the Aβ was freshly prepared, there was ample time (48 hrs) for aggregation and fibrillogenesis to occur. However, it will be important to indentify the conformations of the Aβ that are present in our cultures at different time points and these studies are in progress in our laboratory. As a potential mechanism of GAL neuroprotective effect, it may also be of interest to explore the interaction of GAL and Aβ aggregation.

4.3 GALR2 is involved in the neuroprotective effect of GAL

Considerable studies have shown the GALR mRNA expression in BF. GALR1 mRNA was detected with relative abundance in the BF by northern blot, in situ hybridization and solution hybridization/RNase protection analyses (Counts et al., 2001). In saturation experiments and the comparative radioligand GAL binding studies in human

BF, it is suggested that the radioligands were interacting with different GALRs within the BF and that two receptor subtypes, i.e., GALR1 and GALR2 exist in approximately the same concentration (Mufson et al., 1998). However, the presence of GALR3 proteins in the BF has not been reported yet. Therefore, we tried to identify the GALR1 and GALR2 in the culture by using a laboratory synthesized antibody and a commercially available antibody respectively (Pham et al., 2002). However, we were unable to verify the specificity of these antibodies in rat brain tissue. The GALR1 antibody was only used to label receptors in gastrointestinal tract, not CNS, which might be the reason for the failure of labeling the GALR1 in our BF neuronal cultures (Pham et al., 2002).

Because of the failure to recognize the GALRs by antibody, we tried to explore the involvement of specific GALR in neuroprotective effects by receptor specific compounds. We applied GALR2 specific agonist AR-M1896, a chimeric peptide from AstraZeneca, and showed that this agonist could mimic the neuroprotection of GAL against Aβ toxicity in the primary BF culture measured by live-dead assay. This peptide has also been used to confirm the neuroprotective effects of GAL against staurosporine and Glu-induced cell death in hippocampal cultures (Elliott-Hunt et al., 2004). This peptide binds to GALR2 preferentially with 500-fold specificity over GALR1, and with an almost complete loss of GALR1 activation (Elliott-Hunt et al., 2004; Liu et al., 2001). Hence, we believe that our data suggest the presence of GALR2 in the BF neuronal cultures and the involvement of GALR2 in the neuroprotective effects of GAL against Aβ toxicity.

We also applied a non-specific GALR agonist galnon to mimic the neuroprotective effect of GAL. This low molecular weight agonist was found to displace

^{125}I-galanin with micromolar affinity at Bowes cellular and rat hippocampal membranes (Saar et al., 2002). It is also observed that *in vivo*, i.p. injection of galnon reduced the severity and increased the latency of pentylenetertrazole-induced seizures in mice and reversed the proconvulsant effects of the GALR antagonist M35 (Saar et al., 2002). However, we did not obtain consistent results with galnon at up to micromolar concentration in BF primary cultures, probably because galnon is a non-peptide chemical. Furthermore, it has been reported recently that in CNS, galnon demonstrates both agonist and antagonist properties (Wang et al., 2004), which might also explain the inconsistent results observed with galnon in our BF neuronal culture model.

We did not study the activation of GALR1 or GALR3 because of the lack of receptor subtype-specific agonist. It is of interest to determine if other receptors may also be involved in GAL effects, especially GALR1 since GALR1 mRNA is relatively abundant in the BF (Counts et al., 2001). To investigate the intracellular signaling pathways downstream of specific GALRs may also be helpful to understand the neuroprotective effect we observed. GALR2 is coupled to the activation of phospholipase C and PKC, mediating pertussis toxin-sensitive MAPK activity (Wang et al., 1998). Activation of GALR2 could probably improve cholinergic tone and also the survival of cholinergic BF neurons. It is demonstrated that GALR2 is the principle receptor involved in the initiation and maintenance of axonal regeneration after peripheral nerve injury which is also PKC dependent (Mahoney et al., 2003). In contrast, GALR1 is mainly coupled to G_i protein and inhibition of adenylyl cyclase and therefore potentially may inhibit cholinergic function (Counts et al., 2003). Based on these observations, the different signaling pathways GALRs initiate may explain the

controversies in behavioral studies, transgenic models, and also the indication of the role GAL plays in AD. Therefore the development of GALR specific ligands will be critically important for future research in determining the different physiological and pathophysiological roles of GAL.

Not only the paucity of receptor specific agonists, but also the shortage of GALR antagonists has hampered study of functional role of GAL in AD. There are a few chimeric peptides as candidates for GALR antagonists such as M15, M35, M40 and C7. However, the uncertainty surrounding these peptides is that they act as GALR agonists in some models. A number of studies showed that these chimeric peptides act as agonists *in vitro* (Wang et al., 2004). In CHO cells expressing the GALR 2 receptor, M15, M35, M40 and C7 were found to be full agonists in the stimulation of phosphoinositide hydrolysis (Smith et al., 1997). These peptides also behaved as agonists at the cloned rat GALR1 receptor (Smith et al., 1997; Forray et al., 1996). In our laborotary, M15 (galantide) applied in nM dose range depolarized rat cholinergic BF neurons in much the same manner as GAL (Jhamandas et al., 2002). These putative antagonists will add to the difficulty in evaluating the effects of GAL in the CNS and elsewhere.

4.4 Activation of caspase 3 is involved in Aβ toxicity and GAL neuroprotective effect

Recent studies suggest the upregulation of various caspases (caspase 3, 8, and 9) in the post-mortem tissue of sporadic AD patients (Eckert et al., 2003). In *in vitro* culture models, multiple caspases are activated in Aβ induced neuronal apoptosis, such as caspase 2, 3, 6 and 8 (Stadelmann et al., 1999; Allen et al., 2001; Wei et al., 2002). Here we consistently report the activation of caspase 3 after Aβ treatment in BF

neuronal cultures. When the cultured cells were pretreated with GAL, we observed less caspase activation, by both Western blot and immunostaining. As an executioner caspase, activation of caspase 3 will result in the cleavage of various cellular proteins and lead to the apoptotic cell death (Chang and Yang, 2000). These observations indicated that GAL might protect the culture against Aβ toxicity by inhibition of apoptotic pathways activated by Aβ. Combining the VChAT and cleaved caspase 3 staining, we further demonstrated that GAL protected cholinergic neurons in BF neuronal culture through inhibition of apoptotic cell death.

4.5 Possible mechanisms underlying GAL neuroprotection

There is no direct evidence identifying the major mechanism of GAL neuroprotection against Aβ toxicity. However, the mechanisms of Aβ toxicity have been comprehensively studied, including: 1) increasing Ca^{2+} influx and dysregulation of calcium homeostasis; 2) free radical accumulation; 3) activation of apoptotic intracellular pathway. Towards the mechanisms of Aβ toxicity, we infer that GAL may possible protect cholinergic neuronal cultures through the following mechanisms: 1) Attenuation of calcium-mediated neurotoxicity induced by Aβ. There is evidence showing that GAL reduced Ca^{2+} influx through L- and N-type voltage-sensitive Ca^{2+} channels (Palazzi et al., 1991; Kalkbrenner et al., 1995). GAL could also inhibit glucose-stimulated insulin release by hyperpolarization and lowering of cytoplasmic free Ca^{2+} concentration (Ahren et al., 1986). 2) Affecting free radical generation. There is no direct evidence showing GAL affecting free radical generation. However, GAL has been reported to interact with glutamatergic system and inhibit Glu release (Kinney et al., 1998; Mazarati et al., 2000).

If Glu is involved in Aβ toxicity in AD, it is possible that GAL protects cultured cells through interaction with glutamatergic system. 3) Intracellular pathway. The intracellular signaling pathways coupled to GAL receptor activation have not been well recognized. There is evidence showing that activation of MAPK pathway following GAL receptor binding (Branchek et al., 1998). This seems contradictory to neuropretective effects of GAL since activation of MAPK pathway will lead to tau phosphorylation that will be deleterious to cell survival. However, there may be other survival pathways coupled to GAL receptor activation of G proteins. 4) Interaction with Aβ targeted receptors and Aβ aggregation. There is at present no supporting evidence for this possibility, although further studies in this area may prove fruitful.

4.6 Conclusion

We have demonstrated that GAL is neuroprotective against Aβ toxicity in BF neuronal culture, and GALR 2 may be involved in mediating this neuroprotective effect. The neuroprotection in our model is moderate (15-20%). However, it clearly suggests a neuroprotective rather than a neurotoxic role for GAL. In this respect our findings are novel and distinct. We also demonstrated that the neuroprotective effect of GAL is directed mainly towards improving survival of cholinergic BF neurons rendered dysfunctional by Aβ, which is highly relevant to the neuropathology observed in AD. Therefore, further efforts in this developing field to better understand the role of GAL plays in AD, the underlying mechanisms of action of GAL, and the development of new therapy based on GAL agonists and antagonists would be highly beneficial in treating AD and similar neurodegenerative diseases.

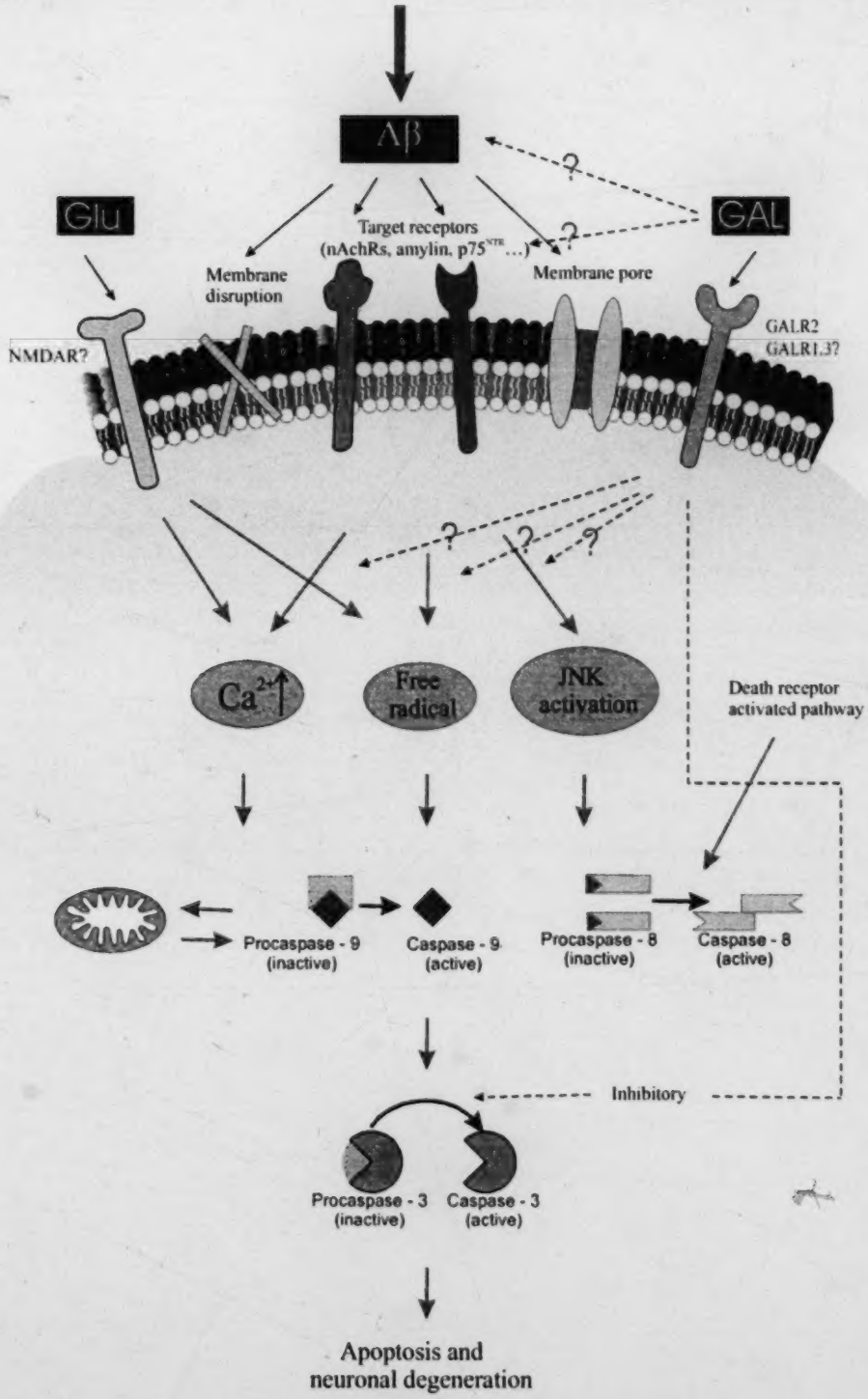

Aβ
?
Glu
GAL
Target receptors
(nAchRs, amylin, p75NTR...)
?
Membrane
disruption
Membrane pore
NMDAR?
GALR2
GALR1,3?
?
?
?
Ca2+↑
Free
radical
JNK
activation
Death receptor
activated pathway
Procaspase - 9
(inactive)
Caspase - 9
(active)
Procaspase - 8
(inactive)
Caspase - 8
(active)
Inhibitory
Procaspase - 3
(inactive)
Caspase - 3
(active)
Apoptosis and
neuronal degeneration

Figure 12: Aβ induced apoptotic cascade and the involvement of GAL as a neuroprotective factor. Aβ causes apoptotic cell death through the interruption of calcium homeostasis, generation of free radicals and activation of apoptotic intracellular pathways. Several Caspases pathways are activated and cells undergo apoptosis. GAL partially inhibits the caspase 3 activation induced by Aβ. However, the mechanisms of the GAL neuroprotective effects remain undiscovered. GALR2 may be involved in mediating GAL neuroprotection.

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